Black Soybean Extract Protects Against TMT-Induced Cognitive Defects in Mice

Ji Hee Jeong,¹ Yu Na Jo,¹ Hyeon Ju Kim,¹ Dong Eun Jin,¹ Dae-Ok Kim,² and Ho Jin Heo¹

¹Division of Applied Life Science, Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Korea.

²Department of Food Science and Biotechnology, Skin Biotechnology Center, Kyung Hee University, Yongin, Korea.

ABSTRACT To find a neuroactive compound with a potent inhibitory effect on acetylcholinesterase (AChE) and *in vivo* anti-amnesic activity from natural resources, we evaluated anthocyanins and nonanthocyanins from black soybean extract. Nonanthocyanins from black soybean extract were the most potent and dose-dependent AChE inhibitors. Intracellular reactive oxygen species accumulation resulting from H₂O₂ treatment was significantly decreased compared with cells treated with H₂O₂ only. Nonanthocyanins were also neuroprotective against H₂O₂ treated neurotoxicity by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assay. Finally, nonanthocyanins from black soybean in the preadministration group attenuated trimethyltin (TMT)-induced memory injury in both *in vivo* tests. AChE, prepared from mice brain tissues, was inhibited by nonanthocyanins from black soybean in a dose-dependent manner. Malondialdehyde generation in the brain homogenates of mice treated with nonanthocyanins from black soybean was decreased. We concluded that nonanthocyanins from black soybean had an efficacious *in vitro* AChE inhibitory activity, and protected against H₂O₂-induced neurotoxicity. In addition, our findings suggest that nonanthocyanins from black soybean may improve the TMT-induced learning and memory deficit because of AChE inhibition of mice brain tissue. Consequently, these results demonstrate that the nonanthocyanins from black soybean could possess a wide range of beneficial activities for neurodegenerative disorders.

KEY WORDS: • *acetylcholinesterase* • *black soybean* • *cognitive function* • *Glycine max* (L.) Merr. • *syngic acid* • *trimethyltin*

INTRODUCTION

SOYBEAN (*GLYCINE MAX*) has been one of the long important nutritional sources since ancient times. Soybeans are extremely versatile and can be made into a variety of foods. Asians including Koreans consume an average of 20 to 80 g of traditional soy foods daily, the most common of which include tofu, miso, and tempeh. Americans consume much less soy, only about 1 to 3 g daily, and this is mostly in processed forms such as soy drinks, breakfast cereals, energy bars, and soy burgers.¹ Black soybean [*Glycine max* (L.) Merr.], which is characterized by a black seed coat and yellow or green cotyledon, has long been consumed worldwide, especially in Korea. In Korea, black soybeans are used to prepare several different traditional fermented foods, including *meju* (soybean cake), *cheonggukjang* (cooked soybean), *kanjang* (soybean sauce), and *doenjang* (soybean paste).² Black soybean is also used as a medicinal food for kidney disease, blood circulation, oxidative stress, and counteracting toxicity because of their various physiological activities.³ In addition, the black hull contains various well-characterized functional polyphenols

such as anthocyanins, procyanidins, and catechins.⁴ Recently, many new findings have demonstrated the health benefits of black soybean. Superoxide dismutase (SOD) can scavenge superoxide radicals (O₂⁻), which can lead to fatal damage to cells, and so it plays a crucial role in the defense against oxidative damage. Wang *et al.*⁵ showed that the black soybean SOD possesses a more efficient protective capacity on cells at a lower dosage, compared with human SOD.

The inhibition or induction of acetylcholinesterase (AChE) activity can cause changes in acetylcholine (ACh) concentration in the body that can lead to adverse effects. Especially, inhibition of the AChE causes high concentrations of ACh to accumulate in the brain, whereas induction of the AChE will cause more hydrolysis of the ACh into acetate and choline, which decreases the concentration of the ACh in the brain.⁶ In other words, AChE hydrolyzes the neurotransmitter ACh at the synaptic cleft of cholinergic synapses and neuromuscular junctions. In addition to its role in cholinergic transmission, AChE is associated with brain development, learning, memory, and neuronal damage. Several “nonclassical” AChE activities have been described as neurite growth, hematopoiesis, and osteogenesis, in addition to an action as adhesion protein in synaptic development and maintenance.⁷ AChE inhibitors from natural resources were previously reported by other scientists. Many natural resources such as *Bacopa monniera* and *Ginkgo biloba* have been proven to

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Address correspondence to: Ho Jin Heo, PhD, Division of Applied Life Science, Institute of Agriculture and Life Science, Gyeongsang National University, Jinju 660-701, Korea.
E-mail: hjher@gnu.ac.kr

have significant AChE inhibitory activity.⁸ The majority of the studies on natural products as inhibitors of AChE have emphasized alkaloids, such as physostigmine and galanthamine. Galanthamine, an alkaloid isolated from different *Galanthus* species (Amaryllidaceae), has been found to be potent and reversible AChE inhibitors.⁹ Natural glycoalkaloids produced by plants of the family Solanaceae, which includes potatoes and tomatoes, inhibit both AChE and butyrylcholinesterase (BuChE).¹⁰

To find a new active constituent with potent AChE inhibitory activity and *in vivo* anti-amnesic activity from natural resources, we investigated the neuroprotective effects of anthocyanin and nonanthocyanin fractions from black soybean extract. Further, AChE activity in the brains of mice and malondialdehyde (MDA) levels of mice brain homogenates after *in vivo* assay were investigated to confirm anti-amnesic effects of nonanthocyanins from black soybean.

MATERIALS AND METHODS

Materials

9-Amino-1,2,3,4-tetrahydroacridine (Tacrine), acetylcholine, 5,5-dithio-bis(2-nitro)benzoic acid (DTNB), trimethyltin (TMT), hydrogen peroxide (H₂O₂), 2',7'-dichlorofluorescein diacetate (DCF-DA), vitamin C, thiobarbituric acid, HEPES, sodium bicarbonate, penicillin, and all other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA). The PC12 cell line (rat pheochromocytoma cells) was obtained from the Korea cell line bank (Seoul, Korea). Roswell Park Memorial Institute (RPMI) 1640 medium was purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). Fetal bovine serum was obtained from Gibco BRL (Grand Island, NY, USA).

Extraction of black soybean

Black soybean was purchased from a local market (Jinju, Korea), in September, 2011, and was authenticated by Institute of Agriculture and Life Sciences, Gyeongsang National University where voucher specimens are maintained. Black soybean was separated into seed coats and beans dehulled by hand. The phenolics in seed coat were extracted from 10 g of seed coats using 70% acetone (200 mL) by the ultrasound-assisted method.¹¹ Phenolic extraction for black soybean seed coat was done in three replications. A simple fractionation of black soybean seed coat extracts was performed using preconditioned C₁₈ Sep-Pak cartridges to separate anthocyanins from nonanthocyanins¹² and then stored -20°C prior to use.

AChE in vitro inhibition assay

The AChE assay was performed according to the colorimetric method of Ellman *et al.*¹³ using acetylthiocholine iodide as a substrate. Protein concentration was determined using the Quant-iT™ protein assay kit (Invitrogen, Carlsbad, CA, USA). The rates of hydrolysis by AChE were monitored spectrophotometrically using a 96-well micro-

plate reader. Each sample (10 µL) was mixed with an enzyme preparation (10 µL) and incubated at 37°C for 15 min. Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture [70 µL; 0.5 mM acetylthiocholine and 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. A blank reaction was measured by substituting saline for the enzyme.

Cell culture

The PC12 cell line was derived from a transplantable rat pheochromocytoma.¹⁴ The cells reversibly respond to nerve growth factor by induction of the neuronal phenotype. Cells (KCLB 21721, Korea Cell Line Bank, Seoul, Korea) were propagated in RPMI 1640 medium containing 10% fetal bovine serum, 50 units/mL penicillin, and 100 µg/mL streptomycin.

Measurement of cellular oxidative stress

Levels of cellular oxidative stress were measured by DCF-DA. PC12 cells were pretreated with various concentrations of nonanthocyanins for 48 h. Cells were then treated with or without H₂O₂ for 2 h. At the end of the treatment, cells were incubated in the presence of 50 µM DCF-DA for 50 min. After incubation, DCF was quantified using a fluorometer (infinite F200; TECAN, Durham, NC, USA) with a 485 nm excitation filter and a 535 nm emission filter. The results were expressed as percent relative to the oxidative stress level of the control cells, which was set to 100%.¹⁵

Determination of cell viability

Cell viability of PC12 cells was evaluated using two different *in vitro* assays: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release into medium assays.¹⁵ For the conventional MTT reduction assay, the amount of MTT formazan product was determined by measuring absorbance using a microplate reader (680; Bio-Rad, Tokyo, Japan) at 570 and 690 nm for test and reference wavelength, respectively.

For the LDH assay, damage to the plasma membrane was evaluated by measuring the amount of the intracellular enzyme LDH released into the medium.

Animals and in vivo experimental design

Institute of Cancer Research (ICR) mice (male, 4 weeks old) were obtained from Samtako (Osan, Korea). The mice were housed two per cage in a room maintained with a 12 h light-dark cycle, 55% humidity, and 24°C ± 1°C temperature. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Gyeongsang National University (certificate: GNU-120409-M0009), and performed in accordance with the Policy of the Ethical Committee of Ministry of Health and Welfare, Korea. Freeze-dried nonanthocyanins from black soybean were mixed in drinking water (5, 10, and 20 mg/kg of body weight). Mice were divided into five groups: (I) control (*n* = 8), (II) TMT (*n* = 8), (III) TMT + nonanthocyanins from

black soybean 5 mg/kg (N5) ($n=8$), (IV) TMT+nonanthocyanins from black soybean 10 mg/kg (N10) ($n=8$), and (V) TMT+nonanthocyanins from black soybean 20 mg/kg (N20) ($n=8$). The mice were allowed free access (*ad libitum*) to water, in which the nonanthocyanins had been dissolved for 3 weeks. On average each mouse consumed 3 mL of drinking water a day in this experiment and drinking water in a cask was changed every 3 days. Amnesia impairment was induced in the mice with TMT given subcutaneously (2.5 mg/kg of body weight).¹⁶

Y-maze test

The Y-maze test was performed 2 days after the TMT injection. The maze was made of black-painted plastic, and each arm of the maze was 33 cm long, 15 cm high, and 10 cm wide, and was positioned at a constant angle. Each mouse was placed at the end of one arm, and allowed to freely move through the maze during an 8 min period. The sequence of arm entries was recorded manually. A spontaneous alternation behavior was defined as entry into all three arms on consecutive choices in overlapping triplet sets.¹⁷ The percentage spontaneous alternation behavior was calculated as the ratio of actual to possible alternations.

Passive avoidance test

A shuttle box was divided into two chambers, one illuminated and one dark, and separated by a guillotine door. The step-through passive avoidance apparatus consisted of a lighted chamber (width: 20 cm length: 20 cm height: 30 cm) made of transparent plastic and a dark chamber whose walls were made of dark opaque plastic (width: 20 cm length: 20 cm height: 30 cm). During the training trial, each mouse was placed in the lighted compartment, and then when the mouse entered the dark compartment the door was closed, and the mouse received an inescapable electric shock (0.5 mA, 1 s). The test trial was given 1 day after the training trial, and again the mouse was placed in the lighted compartment and the latency time to enter the dark compartment was measured (the step-through latency maximum testing limit was 300 s).¹⁵

Preparation of tissue samples

For biochemical studies, mice were sacrificed. Brains were dissected and kept at -70°C before use. Whole brains were homogenized with 10 vol. of cold phosphate buffered saline in an ice bath. Homogenates were directly centrifuged 1000 g for 10 s (twice with 30 s interval) to obtain the supernatant. Supernatant aliquots were used to determine brain MDA levels, AChE activity, and protein content.

Determination of ex vivo AChE activity

The AChE assay was performed according to the colorimetric method of Ellmans *et al.*¹³ using acetylthiocholine iodide as a substrate. Whole brains homogenate (5 μL) was mixed with 65 μL of sodium phosphate buffer (50 mM, pH

8.0) and the mixtures incubated at 37°C for 15 min. Absorbance at 405 nm was read immediately after adding an Ellmans's reaction mixture [70 μL ; 0.5 mM acetylthiocholine and 1 mM 5,5'-dithio-bis(2-nitrobenzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. The results were expressed as percent relative to the activity of the control group (100%).

MDA levels

The MDA level was assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation. In brief, 160 μL of each homogenate was mixed with 960 μL of phosphoric acid (1%, v/v) followed by addition of 320 μL thiobarbituric acid solution (0.67%, w/w). The mixture was incubated at 95°C in a water bath for 1 h. After cooling, the colored complex was separated by centrifugation (1600 g for 10 min), and absorbance was measured at 532 nm using tetramethoxypropane as a standard. MDA levels were expressed as nmol/mg of protein.¹⁵

High-performance liquid chromatography analysis

Phenolic compounds were measured at 280 nm using a photo diode array detector (Ultimate 3000 series, Dionex, CA, USA). Separation was achieved with a ZORBAX Eclipse plus C₁₈ column (250 mm $\times$ 4.6 mm id, 5 μm ; Agilent Technologies, Inc., Santa Clara, CA, USA). The mobile phase consisted of water with 0.1% acetic acid (A) and methanol with 0.1% acetic acid (B). The linear gradient was 0–100% (B) in 60 min, at a flow rate of 1.0 mL/min and the injection volume was 20 μL . Compounds were detected by monitoring the elution at 280 nm. Identification of the phenolics was carried out by comparing their retention times to those of standards.

Statistical analysis

All data were expressed as mean $\pm$ SD. Each experimental set was compared by one-way analysis of variance (ANOVA) and Duncan's multiple-range test ($P < .05$) using SAS software (SAS Institute, Inc., Cary, NC, USA).

RESULTS

Effect of anthocyanins and nonanthocyanins from black soybean on in vitro AChE activity

In this experiment, anthocyanins and nonanthocyanins from black soybean were tested for AChE inhibitory activity using Ellman's colorimetric method.¹³ AChE inhibition assay is utilized to screen the potent AChE inhibitor from edible resources such as black soybean extract (Fig. 1). Nonanthocyanins from black soybean efficiently inhibited effect AChE in PC12 cells. However, the anthocyanins showed lower inhibitory activities and the AChE inhibition was far below 50% at all concentrations tested. The results indicated that nonanthocyanins had more significant inhibitory effects with increasing concentration in the range of 31–250 $\mu\text{g/mL}$. Therefore, there is a need to perform

FIG. 1. Inhibitory effect of phenolics from black soybean against acetylcholinesterase (AChE). The AChE inhibition was expressed as a percentage of enzyme activity inhibited compared with the control value. Values represent the mean $\pm$ SD ($n=3$). ^{abcde}Different superscripts indicate significant difference among groups at $P < .05$.

additional research on the exact AChE inhibition mechanism of nonanthocyanins from black soybean containing active substances. In addition, the IC_{50} value for cellular AChE activity inhibited by the nonanthocyanins was at the level of 233.68 μ g/mL.

Measurement of cellular oxidative stress

Levels of cellular oxidative stress were measured using DCF-DA.¹⁵ Intracellular reactive oxygen species (ROS) accumulation resulting from H_2O_2 treatment was significantly decreased when cells were treated with nonanthocyanins than with those treated with H_2O_2 only (Fig. 2). At the level of 200 μ M vitamin C, as a positive control, decreased oxidative stress about 25% below that of only H_2O_2 treatment group. The nonanthocyanins treatment (15–250 μ g/mL) resulted in a 122–103% decrease of cellular ROS levels compared with the H_2O_2 exposure. These results suggest that the nonanthocyanins protected PC12 cells from oxidative toxicity.

Neuroprotective effects of nonanthocyanins from black soybean

H_2O_2 caused a decrease in cell viability (about 68%), but pretreatment cells with increasing concentrations of the nonanthocyanins inhibited H_2O_2 -induced neurotoxicity (Fig. 3A). The cell viabilities of cells with the nonanthocyanins were about 35–77% at concentrations ranging from 15 to 125 μ g/mL. Especially, the group with ≥ 125 μ g/mL showed more effective protection than vitamin C as a positive control. The results in this study suggest that neuron-like PC12 cell protection by nonanthocyanins from black soybean may be due to the mitochondrial protection mechanisms.

FIG. 2. Effect of nonanthocyanins from black soybean on reactive oxygen species production determined in the presence and absence of H_2O_2 in PC12 cell. Each value represents the mean $\pm$ SD of triplicates. ^{abcde}Different superscripts indicate significant difference among groups at $P < .05$.

Another assay in PC12 cell membrane was performed to confirm the neuroprotective effect of the nonanthocyanins. LDH assay is a means of measuring either the number of cells via total cytoplasmic LDH or membrane integrity as a function of amount of cytoplasmic LDH released into the medium.¹⁸ In this LDH assay, H_2O_2 as an oxidative cytotoxicant increased plasma membrane damage in PC12 cells, whereas the nonanthocyanins efficaciously protected the membrane loss. In other words, as shown in Figure 3B, H_2O_2 treatment caused an increase in LDH release into the medium (35%), but the nonanthocyanins treatment efficiently inhibited cytoplasmic enzyme release.

Effect of the nonanthocyanins on Y-maze test and passive avoidance test

To confirm the attenuating effect of the nonanthocyanins against TMT-induced learning and memory impairment, the Y-maze and passive avoidance test were utilized. The Y-maze test was carried out 2 days after TMT-injection. Mice were treated with the nonanthocyanins in the drinking water at various concentrations for up to 3 weeks prior to TMT administration. The nonanthocyanins ameliorated TMT-induced learning and memory deficit. The TMT-injected mice exhibited a significantly impaired spatial working memory (20% decrease in alternation behavior) when compared with that of the control group (Fig. 4A). Treatment with the nonanthocyanins increased spontaneous alternation behavior in the TMT-injected mice. In contrast, the number of arm entries did not change among all the experimental groups (Fig. 4B).

In the passive avoidance performance tests, the TMT-injected mice showed significantly shorter latency time during the retention trials, with a 61% decrease in step-through latency compared with the control group. Treatment with the nonanthocyanins attenuated the TMT-treated

FIG. 3. Cell protective effect of nonanthocyanins from black soybean on H₂O₂-induced neurotoxicity in PC12 cell. **(A)** PC12 cells were pretreated for 48 h with various concentrations of nonanthocyanins. After 48 h, the cells were treated with 200 μM H₂O₂ for 3 h by MTT reduction assay. **(B)** Inhibition of lactate dehydrogenase release of the nonanthocyanins on H₂O₂-induced membrane injury in PC12 cell. Each value represents the mean ± SD (*n* = 3). ^{abcdef}Different superscripts indicate significant difference among groups at *P* < .05.

impairment of mice in the passive avoidance test (Fig. 5). The survival rate of experimental mice was 100% (data not shown). The above *in vivo* results suggest that 3 weeks of pretreatment with the nonanthocyanins effectively prevented drug-induced decrement in learning and memory impairment. Both *in vivo* results suggest that a diet containing nonanthocyanins of black soybean may protect against drug-induced cognitive loss.

Effect of the nonanthocyanins on brain AChE activity and lipid peroxidation level in TMT-injected mice brain

After *in vivo* assays, AChE activity in the mouse brain was determined to investigate the physiological effect of

FIG. 4. Effect of nonanthocyanins from black soybean on spontaneous alternation behavior in trimethyltin (TMT)-induced amnesic mice. The Y-maze test was performed 2 days after the TMT injection (2.5 mg/kg of body weight). Sample groups (N5, N10, and N20) were injected with TMT followed by feeding with the nonanthocyanins (5, 10, and 20 mg/kg of body weight, respectively). The spontaneous alternation behavior **(A)** and number of arm entries **(B)** were measured over 8 min. Results shown are mean ± SD (*n* = 8). ^{ab}Values with the same letter are not significantly different (*P* < .05).

nonanthocyanins. As shown in Figure 6, TMT-treated group showed increased AChE activity, whereas the pretreatment groups with the nonanthocyanins relatively inhibited AChE in the brains of mice. AChE activity of N20 group was significantly decreased when compared with that of the TMT-treated group.

We also examined whether the nonanthocyanins could inhibit TMT-treated lipid peroxidation by measuring the levels of MDA, a marker of lipid peroxidation, in the brain homogenates. The levels of MDA were increased (0.2 nmole/mg protein) in the TMT-treated group when compared with that of the control group. The nonanthocyanins administration significantly lowered MDA concentrations in a dose-dependent manner (Fig. 7).

FIG. 5. Effect of nonanthocyanins from black soybean on TMT-induced memory impairment mice. Passive avoidance test was performed to check the step-through latency for 300 s. Each behavioral test was conducted 3 days after TMT injection (2.5 mg/kg of body weight). Sample group (N5, N10, and N20) were injected with TMT followed by feeding with nonanthocyanin fraction from black soybean extract (5, 10, and 20 mg/kg of body weight, respectively). The results shown are mean $\pm$ SD ($n=8$). ^{abcd}Different superscripts indicate significant difference among groups at $P<.05$.

High-performance liquid chromatography analysis of the nonanthocyanins

Nonanthocyanins from black soybean were analyzed for their phenolic composition by high-performance liquid chromatography (HPLC). The nonanthocyanins contained

FIG. 6. Effect of nonanthocyanins from black soybean on AChE activity from TMT-induced mice brain homogenate. Sample group (N5, N10, and N20) were injected with TMT followed by feeding with nonanthocyanin fraction from black soybean (5, 10, and 20 mg/kg of body weight, respectively). The results shown are mean $\pm$ SD ($n=8$). ^{abc}Different superscripts indicate significant difference among groups at $P<.05$.

FIG. 7. Effect of nonanthocyanins from black soybean on lipid peroxidation in TMT-induced mice brain homogenate. Sample group (N5, N10, and N20) were injected with TMT followed by feeding with nonanthocyanin fraction from black soybean (5, 10, and 20 mg/kg of body weight). The results shown are mean $\pm$ SD ($n=8$). ^{abc}Different superscripts indicate significant differences among groups at $P<.05$.

various phenolic compounds (Fig. 8). By comparing the retention time and UV spectra of these compounds with those of standards, syringic acid, catechin, and procatechuic acid were detected as main phenolics. Therefore, the results suggest that above three main phenolics may contribute to the neuronal cell protective and learning and memory effects of nonanthocyanins from black soybean seed coat.

DISCUSSION

AChE inhibitors are substances that inhibit the enzyme from breaking down ACh, and thereby increasing both the level and duration of action of the neurotransmitter ACh. Such substances occur naturally as venoms and poisons. Importantly, synthetic inhibitors are used as medicines to treat neurodegenerative disorders such as Alzheimer's disease (AD).¹⁹ Pharmacological treatment of AD is currently based on AChE inhibitors, such as tacrine, donepezil, rivastigmine, and galantamine, which maintain high levels of ACh at the muscarinic and nicotinic receptors in the central nervous system, exerting beneficial effects on cognitive, functional, and behavioral symptoms of the disease.²⁰ Therefore, AChE inhibition assay is utilized to screen for potent AChE inhibitors from edible resources such as black soybean seed coat. Nonanthocyanins from black soybean seed coat showed efficient inhibitory effects against AChE from PC12 cells. However, the anthocyanins showed lower inhibitory activities and the AChE inhibition was far below 50% at all concentrations tested. Thus, there is a need to perform additional research on the exact AChE inhibition mechanism of nonanthocyanins from black soybean seed coat containing bioactive substances.

High ROS levels induce oxidative stress, which can result in a variety of biochemical and physiological lesions. Such

FIG. 8. High-performance liquid chromatography chromatograms of standards (**A**) and the nonanthocyanins (**B**) at 280 nm. (**A**) Retention time 7.205 min, protocatechuic acid; 9.610 min, catechin; 12.078 min, syringic acid. Color images available online at www.liebertpub.com/jmf

cellular damage often impairs metabolic function, and leads to cell death.²¹ The treatment with 200 μ M H_2O_2 significantly increased the intracellular ROS levels (Fig. 2). However, the nonanthocyanins inhibited the H_2O_2 -induced ROS generation. These results suggest that the nonanthocyanins protected PC12 cells from oxidative toxicity. H_2O_2 is also known to increase free radical production in PC12 cells, leading to apoptosis and cell death. It has been shown that oxidative damage causes increased injury in neuronal cell membranes and mitochondrial DNA, and decreases activities of antioxidant enzymes in

neuronal cells.²² Consequently, to further evaluate oxidative stress-induced neurotoxicity, an MTT reduction assay was used. MTT dye reduction assay is based on the catalytic activity of some metabolic enzymes in intact mitochondria,²³ and mitochondria may be one of the most sensitive primary targets of oxidative injury in neuronal cells.²⁴ Therefore, defective mitochondria may stimulate neurodegeneration in AD. The results in this study suggest that neuron-like PC12 cell protection by nonanthocyanins from black soybean seed coat may be due to the mitochondrial protection mechanisms.

Lipid peroxidation is increased in neurodegenerative diseases such as AD. Polyunsaturated fatty acid levels, especially arachidonic acid and docosahexaenoic acid, are high in neuronal cells of brain tissue. They are more sensitive to attack by ROS, peroxidation of which can lead to changes in membrane integrity and cytoplasmic homeostasis.²⁵ As the neuronal plasma membrane is vulnerable to oxidative stress, we measured the LDH activity released from apoptotic PC12 cells into the medium. A quantitative analysis of LDH activity can determine the percentage (%) of dead cells. The nonanthocyanins showed that the pattern of neuronal cell protection was similar to MTT assay (Fig. 3B). These results demonstrate that the nonanthocyanins can protect PC12 cells against the lesion induced by oxidative stress-induced neurotoxicity. Finally, the above data suggest that PC12 cell protection by the nonanthocyanins is partially due to the mitochondrial and cell membrane protective mechanisms on H₂O₂-induced neurotoxicity.

TMT, a potent neurotoxicant, has been shown to cause damage to the limbic system. The neurodegenerative effect of TMT is the most evident due to pyramidal cell loss in the hippocampus leading to various behavioral and biochemical deficits.¹⁶ Our previous research observed that pretreatment with the ethyl acetate fraction from chestnut inner skin increased spontaneous alternations in behavior in A β -injected mice (5 mg/kg of the sample: 91%; 10 mg/kg of the sample: 98%; 20 mg/kg of the sample: 104%).²⁶ When comparing those data with this study, *in vivo* data showed that the groups pretreated with the nonanthocyanins from black soybean increased spontaneous alternation behavior after TMT-induction (5 mg/kg: N5, 97.36%; 10 mg/kg: N10, 103.21%; 20 mg/kg: N20, 106.02%). As shown in Figures 4 and 5, mice treated with the nonanthocyanins also exhibited attenuated TMT-induced impairment in a dose-dependent manner. Both *in vivo* results suggest that a diet containing nonanthocyanins of black soybean seed coat may protect against drug-induced cognitive loss. These data suggest that the TMT-induced cognitive deficit that was exerted via various cytotoxicities including oxidative stress and disruption of hippocampal network activity and others²⁷ could be suppressed by pretreatment with the nonanthocyanins from black soybean.

Although the mechanisms by which TMT induces degeneration are not yet well understood, some researchers suggest that the cholinergic system of synapses in the hippocampus may be damaged by oxidative stress and apoptotic or necrotic cell death with TMT.²⁷ Woodruff and Baisden²⁸ have shown an increase in AChE staining in the dentate gyrus following TMT exposure. In Figure 6, TMT exposed group showed an increase in AChE activity, whereas the treatment of the nonanthocyanins effectively inhibited AChE activity from the brains of mice. Consequently, to accurately describe the relationship between brain AChE inhibition and cognitive performance in mice treated with the nonanthocyanins, the brain region-specific effects of phenolics in the nonanthocyanins need to be clarified in future research.

Another possible mechanism underlying the beneficial effects of nonanthocyanin, other than anti-AChE activity, is its anti-oxidative capacity. It has been reported that a dose of

TMT similar to that used in this study (3 mg of TMT/kg of body weight) elevated ROS in the hippocampus and frontal cortex.²⁹ This is in good agreement with our observations. A product of lipid peroxidation (*i.e.*, MDA) was significantly increased in the group treated with TMT only, whereas in the N20 group it was significantly decreased compared with the TMT-treated mice (Fig. 7). Several studies have found lipid peroxidation changes in brain tissue, including elevated MDA.^{29,30} These oxidative modifications of lipids are not only detrimental themselves, but their end products (*e.g.*, acrolein, 4-hydroxynoneanal and MDA) are also reactive.²⁰ Hence, one should not dismiss the possibility that the nonanthocyanins attenuated the toxicity of TMT through its action as an antioxidant in the brain tissue.

A previous report demonstrated that various parts of black soybean have physiologically active phenolics.³ Xu and Chang³¹ reported that the contents of such phenolics as protocatechuic acid, protocatechualdehyde, and caffeic acid are higher in black soybean than in yellow and other soybeans. Protocatechuic acid and caffeic acid are known to inhibit collagen-induced platelet aggregation at relatively higher concentrations. However, anthocyanins of black soybean have shown weak or no inhibitory effects on platelet activation or aggregation.³²

Different parts of black soybeans have been found to contain different individual phenolic compounds. The seed coats possessed higher contents of protocatechuic acid, vanillic acid, caffeic acid, *m*-coumaric acid, and *o*-coumaric acid than dehulled beans, while dehulled beans possessed higher contents of gallic acid, chlorogenic acid, *p*-coumaric acid, and *trans*-cinnamic acid than seed coats. In addition, several minor phenolic acids were detected in dehulled beans such as *p*-hydroxybenzoic acid, vanillin, and sinapic acid, and in seed coats such as 2,3,4-trihydroxybenzoic acid.³⁰ The results of HPLC analysis were partially consistent with the previous report that nonanthocyanins from black soybean seed coat included protocatechuic acid, catechins, and syringic acid as main phenolics.

These *in vitro* and *in vivo* studies showed that nonanthocyanins from black soybean had an efficacious *in vitro* AChE inhibitory effect, and protective effects against H₂O₂-induced neurotoxicity and TMT-induced cognitive deficits. After completing the *in vivo* behavioral test, the biochemical markers in the brain tissues of the mice also confirmed protective effects of nonanthocyanins from black soybean. Taken together, the results of this study indicate that the nonanthocyanins are potent AChE inhibitors that may be beneficial in treating or slowing the progression of neurodegenerative disease such as AD by restoring neurotransmitter systems and protection against oxidative damage. Therefore, black soybean extract including various phenolics has the potential to benefit healthy people and contribute to prevention of cognitive decline during aging and neurodegenerative disease.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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